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In Vivo and In Vitro Measurement of Brain Phosphodiesterase 4 in Rats After Antidepressant Administration

MASAHIRO FUJITA,[1]* MASAO IMAIZUMI,[1] CARROL D'SA,[2] SAMI S. ZOGHBI,[1] MATTHEW S. CRESCENZO,[1] JINSOO HONG,[1] JOHN L. MUSACHIO,[1] ANTONY D. GEE,[3] JURGEN SEIDEL,[4] MICHAEL V. GREEN,[4] VICTOR W. PIKE,[1] RONALD S. DUMAN,[2,5] AND ROBERT B. INNIS[1]

[1]*Molecular Imaging Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland*

[2]*Department of Psychiatry, Yale University, New Haven, Connecticut*

[3]*Translational Medicine and Technology, PET Division, GlaxoSmithKline, Addenbrooke's Hospital, Cambridge, United Kingdom*

[4]*Trident Imaging, Inc., Rockville, Maryland*

[5]*Department of Pharmacology, Yale University, New Haven, Connecticut*

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ABSTRACT Based largely on in vitro measurements, the mechanism of several antidepressant treatments is thought to involve upregulation of 3′-5′-cyclic adenosine monophosphate (cAMP) signal transduction cascade and a corresponding increase in phosphodiesterase (PDE) 4, the enzyme that metabolizes cAMP. To assess the in vivo status of PDE4, rats were chronically treated with imipramine and then studied with: (1) in vivo positron emission tomography (PET) measurement of (*R*)-[^{11}C]rolipram binding, (2) in vitro measurement of [^{3}H]rolipram binding in brain homogenates, and (3) Western blotting for protein levels of PDE4 isoforms. Imipramine administration caused no significant change in B_{max}/K_d, for both in vivo measurements with (*R*)-[^{11}C]rolipram and in vitro measurements with [^{3}H]rolipram in frontal cortex, hippocampus, and diencephalon. None of 10 isoforms of PDE4A, B, and D measured with immunoblots of frontal cortex and hippocampus showed a significant change. In summary, using relatively large brain regions for both in vivo imaging and in vitro measures of radiolabeled ligand binding and protein levels, chronic imipramine treatment via continuous mini-pump administration caused no significant change in PDE4 levels. Most, but not all, prior in vitro studies have found increased PDE4 levels after antidepressant administration. The current results raise questions about the in vivo effects of antidepressant treatment on PDE4 and on other potentially important experimental factors (e.g., continuous infusion vs. intermittent injection of antidepressant) in large brain areas. However, the results do not deny possibility of changes in discrete areas, which were not studied in the current study applying PET. **Synapse 61:78–86, 2007.**

INTRODUCTION

The mechanism of action for several antidepressant treatments may involve the upregulation of the 3′-5′-cyclic adenosine monophosphate (cAMP) cascade (Duman et al., 1997). This hypothesis is based on rodent studies consistently reporting upregulation of the cAMP cascade induced by chronic but not acute antidepressant treatments. This upregulation extends to several components of the cascade: coupling of stimulatory G-protein and adenylate cyclase (Ozawa and Rasenick, 1991), cAMP-dependent protein kinase (Nestler et al., 1989), transcription factor cAMP response element binding protein (CREB) (Nibuya et al., 1996), and brain derived neurotrophic factor (Nibuya et al., 1995).

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*Correspondence to: Masahiro Fujita, MD, PhD, Building 1, Room B3-10, 1 Center Drive, MSC-0135, Bethesda, MD 20892-0135, USA.
E-mail: masahiro.fujita@nih.gov

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Among components of the cAMP cascade, phosphodiesterase 4 (PDE4), which metabolizes cAMP to 5′-AMP, may be a target of antidepressants. Repeated antidepressant treatment increased PDE4 activity, mRNA and protein levels (Andersen et al., 1983; D'Sa et al., 2005; Suda et al., 1998; Takahashi et al., 1999; Ye et al., 1997, 2000; Zhao et al., 2003a), while one study reported mixed results (Miro et al., 2002). This upregulation may represent a compensatory increase of cAMP metabolism in response to increased cAMP tone. If the mechanism of action for antidepressant treatments involves an upregulation of the cAMP cascade, then inhibitors of PDE may have antidepressant efficacy. Consistent with this theory, rolipram, an inhibitor of PDE4, was reported to induce antidepressant effects in depressed patients similar to amitriptyline or imipramine (Eckmann et al., 1998; Hebenstreit et al., 1989).

The positron emitting labeled version of the active isomer of rolipram (i.e. (*R*)-[^{11}C]rolipram) is an excellent in vivo probe to measure PDE4 levels in brain (Lourenco et al., 1999, 2001; Parker et al., 2005). Although not commonly done in rodents, we implemented a fully quantitative method to measure PDE4 levels in rats with (*R*)-[^{11}C]rolipram, which entails dynamic PET imaging and serial arterial measurements of parent tracer separated from radiometabolites (Fujita et al., 2005). In the present study, we used this method to measure the effect of chronic antidepressant administration on the in vivo levels of PDE. Since the radioligand is given at low mass doses that reversibly bind to only a small percentage of the enzyme (i.e. tracer condition), the ratio of the concentration of ligand bound to the free concentration of parent ligand in plasma equals the ratio of B_{max} to K_d (binding potential (BP), *BP*) (Mintun et al., 1984).

The activity of PDE4 is regulated by phosphorylation (Conti et al., 1995), and studies using recombinant DNAs showed that the phosphorylation also alters binding affinity of rolipram (Hoffmann et al., 1998; Sette and Conti, 1996). We performed both in vivo and in vitro measurement because the phosphorylation status is unlikely to be preserved in processing of the tissue for homogenate binding. PDE4 consists of four independently coded subtypes, PDE4A, B, C, and D, all of which have high affinity binding for rolipram without clear selectivity (Houslay, 2001). To assess the expression of the individual isoforms, we also measured protein levels of PDE4A, B, and D, the three isoforms expressed in brain, by Western blotting.

MATERIALS AND METHODS

Imipramine administration

All animal experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals and were approved by National Institute of Mental Health and Yale University School of Medicine Animal Care and Use Committees. Male Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY). Animals were housed in a temperature- and light-controlled room (22–24°C; 6:00 am to 6:00 pm, respectively) and were allowed free access to food pellets and water. Imipramine ((16.3 ± 0.4) mg/kg/day, with these and subsequent data expressed as mean ± SEM; Sigma-Aldrich, St. Louis, MO) was administered subcutaneously for 20 ($n = 13$) or 27 ($n = 3$; 1 of the 3 for PET and 2 for Western blotting) days using osmotic minipump (Alzet, Cupertino, CA). In previous studies, similar doses of imipramine upregulated PDE4 (Andersen et al., 1983; Suda et al., 1998). At the end of the administration, proper infusion was confirmed in all animals by taking out and visually inspecting the fluid inside of the pump. The dose was based on the average body weight at the start (268 ± 3 g) and the end (345 ± 10 g) of the treatment. For control animals, saline was administered during the same period using osmotic minipump. Because it was not possible to perform all of the following experiments in each animal, they were separated into three sets, each composed of saline- and imipramine-treated groups. One set of animals ($n = 5$ for saline and $n = 5$ for imipramine) was used for PET and subsequently for homogenate binding assays. A second set ($n = 5$ for saline and $n = 5$ for imipramine) was used for the measurement of plasma free fraction (f_1) of (*R*)-[^{11}C]rolipram. A third set ($n = 6$ for saline and $n = 6$ for imipramine) was used for Western blotting.

PET with measurement of arterial input function

At the end of saline ($n = 5$) or imipramine ($n = 5$) treatment, PET experiments were performed as described previously under isoflurane anesthesia by measuring (*R*)-[^{11}C]rolipram in both brain and arterial plasma in each experiment (Fujita et al., 2005). To measure total distribution volume, V'_T ($= f_1(B_{max}/K_d$ + nondisplaceable activity); B_{max}: binding site density, K_d: dissociation constant of (*R*)-[^{11}C]rolipram), simultaneous measurement is required for brain and arterial plasma. Our previous experiment showed that nearly all of the brain activity was (*R*)-[^{11}C]rolipram but not radiolabeled metabolites. (*R*)-[^{11}C]rolipram was administered i.v. over 6 min, with a dose (MBq) of 81 ± 6 in saline and 79 ± 6 in imipramine groups. The specific activity (GBq/μmol) was 70 ± 3 in saline and 71 ± 4 in imipramine groups. The injected mass (pmol/g body weight) was 3.10 ± 0.27 in saline and 3.35 ± 0.27 in imipramine groups. There were no significant differences in any of these measures. PET images were acquired for 60 min using the Advanced Technology Laboratory Animal Scanner (ATLAS) equipped with lutetium gadolinium oxyorthosilicate doped with cerium (LGSO)/gadolinium oxyorthosi-

licate doped with cerium (GSO) phoswich detector modules (Seidel et al., 2003). Reconstruction of the PET data with 3D exact positioning ordered subset expectation maximization algorithm achieved a 1.7-mm full width half maximum at the center. (Johnson et al., 2002; Liow et al., 2003). Because the reanalysis of previously acquired data (Fujita et al., 2005) showed that data acquisition for 60 min was adequate, PET imaging was performed for 60 rather than 80 min. Total (free + protein bound) (*R*)-[^{11}C]rolipram levels were measured in arterial plasma using radio-high-performance liquid chromatography (HPLC) by obtaining samples at 10 time points by applying the method described previously (Zoghbi et al., 2006).

Measurement of f_1 of (*R*)-[^{11}C]rolipram

Because of the short half-life of carbon-11 ($t_{1/2} = 20.4$ min), it was not possible to measure radioactive metabolites in plasma and f_1 in the same experiment. Therefore, f_1 was measured in separate experiments without PET imaging using saline- ($n = 5$) and imipramine- ($n = 5$) administered animals. From these animals, 2 ml blood was drawn from the heart under isoflurane anesthesia, and f_1 was measured by ultrafiltration as previously described (Gandelman et al., 1994). $f_1 \times$ total (*R*)-[^{11}C]rolipram is free (*R*)-[^{11}C]rolipram level.

Homogenate binding assays

At the end of each PET scan, samples of frontal cortex, hippocampus, and diencephalon were obtained for in vitro homogenate binding using [^{3}H]rolipram (Amersham Biosciences, Piscataway, NJ). Frontal cortex and hippocampus were sampled because previous studies consistently showed antidepressant-induced upregulation of the cAMP cascade in these regions. Diencephalon was also sampled because our previous work showed slightly faster association and dissociation of (*R*)-[^{11}C]rolipram binding in this region than other regions (Fujita et al., 2005). Thalamus and hypothalamus were combined for the diencephalon region to reduce partial volume effects in the PET measurement. [^{3}H]rolipram binding was measured as described previously (Zhao et al., 2003b) with small modifications. In brief, the tissue was homogenized in binding buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5), and the membrane and the cytosolic fractions were separated by centrifugation at 15,000 g for 15 min. Tissue preparations containing 125 μg protein were incubated in duplicate at 30°C in 500 μl buffer containing 0.5 nm [^{3}H]rolipram. Nonspecific binding was defined in the presence of 40 μM nonradioactive rolipram (Sigma-Aldrich, St. Louis, MO). Reactions were stopped after 1 h by adding 5 ml ice-cold binding buffer and rapid vacuum filtration through GF-B glass fiber filters (Brandel, Gaithersburg, MD) that had been soaked in 0.3% polyethyleneimine. Protein concentrations of the membrane and the cytosolic fractions were measured using a dye reagent kit (BCA™ Protein Assay Kit, Pierce, Rockford, IL) and bovine serum albumin as a standard.

Western blotting

Another set of saline- ($n = 6$) and imipramine- ($n = 6$) treated animals were used for Western blot analysis of the hippocampus and frontal cortex as previously described (Takahashi et al., 1999). The dissected hippocampus and frontal cortex were frozen on dry ice and then homogenized (50 mg wet weight/ml) in 1% SDS. The samples were diluted in loading buffer to a final concentration of 50 mM Tris, pH 6.7, 4% glycerol, 4% SDS, 2% b-mercaptoethanol, and bromophenol blue as a marker. Each sample (10 – 40 μg of protein) was subjected to SDS-PAGE. Proteins were subjected to electrophoretic transfer to nitrocellulose filters, and the resulting filters were incubated with PDE4 specific antibodies in 10 mM phosphate buffer, pH 7.2, 140 mM NaCl, and 0.05% Tween 20 containing 2% milk for blocking. Antibodies selective for each of the three PDE4 subtypes (FabGennix Inc, Frisco, TX) were used per the vendor's specifications. In the present study, we have confirmed the antibody specificity for individual PDE4 isoforms using tissue from PDE4A and PDE4B null mutant mice (data not shown). For visualization of the PDE4 bands, the filters were incubated with goat antirabbit antibody (1:2000) conjugated with horseradish peroxidase (Vector Laboratories, Burlingame, CA). The filters were then developed using the enhanced chemiluminescence system and exposed to Hyperfilm (GE Healthcare UK, Buckinghamshire, UK). The resulting bands were quantified by outlining each of the PDE4 isoform using the NIH Image 1.62 analysis program.

Data analysis

As described previously (Fujita et al., 2005), in PET experiments, V'_T was measured in frontal cortex, hippocampus and diencephalon (Fig. 1) by obtaining arterial input function in each experiment and by applying nonlinear least squares fitting with an unconstrained two-compartment model using PMOD 2.65 (Burger et al., 1998). Brain and blood data of PET scans were expressed as % standard uptake value (SUV) by normalizing with injection activity and body weight using the formula of %SUV = [activity in brain or plasma]/[injection activity] × 100 × body weight (g). To avoid the variability of drawing regions among animals, a single set of regions were drawn on MRI to which an average PET image created from all frames of a single animal was coregistered (Fig. 1). PET data of each animal were coregistered to the PET image used for the MR coregistration. Coregistrations were performed using the 12

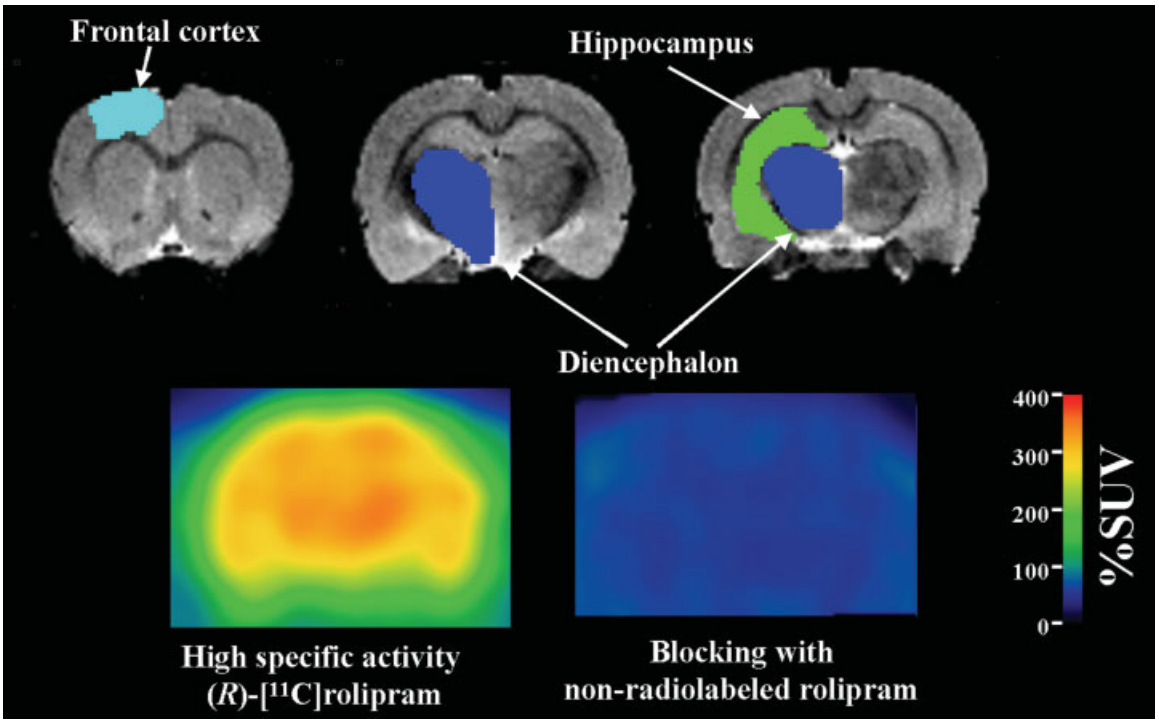

Fig. 1. MR images taken from Schweinhardt et al. (2003) showing studied regions (upper), (*R*)-[^{11}C]rolipram PET without (lower left), and with a binding site saturating dose of nonradiolabeled rolipram (3.9 nmol/g body weight, lower right). The left PET image is from an imipramine-treated animal used in this study and the right one is from a previous study (Fujita et al., 2005). MR images are displayed from rostral (left) to caudal (right) direction. The PET images are in the same space as the middle of the three MR images and were created by integrating all frames. The PET intensity was normalized to injected dose and body weight. A value of 100% standardized uptake value (SUV) is equal to the concentration of radioligand that would be achieved if it were uniformly distributed in the body. Comparison between PET images with and without blocking indicates that the majority of the PET signal was from specific binding of (*R*)-[^{11}C]rolipram. The fairly uniform distribution of specific binding is compatible with the known distribution of PDE4s.

parameter model of Oxford University Centre of Functional Magnetic Resonance Imaging of the Brain Linear Registration Tool (FLIRT) (Jenkinson and Smith, 2001) implemented in MEDx 3.4 (Medical Numerics, Sterling, VA). The volume of the regions was 56, 132, and 121 mm^3 for frontal cortex, hippocampus, and diencephalon, respectively. Percentages of standard errors of nonlinear least-squares estimation relative to the estimated values (coefficient of variation, %COV) were calculated for V'_T from the covariance matrix (Carson, 1986) using the generalized form of error propagation equation (Bevington and Robinson, 2003) where correlations among rate constants (K_1, k_2, k_3, and k_4) were taken into account.

In vitro homogenate binding measurements express K_d relative to the free concentration of radioligand. For in vivo studies, the comparable measurement would use the free level of radiotracer in plasma (F)—that is, that which is not bound to protein. For studies conducted at low occupancy, $B/F = B_{max}/K_d = BP$. However, these equations apply only when F is the free level of tracer and not its total concentration (i.e. free plus protein bound). For this study that involves comparison with standard in vitro receptor binding methods, we will use BP to refer to BP expressed relative to the free concentration of radioligand in plasma. V_T ($=V'_T/f_1 = BP$ + nondisplaceable activity) will be reported to compare in vivo and in vitro measurements.

In homogenate binding assays, B_{max} and K_d of [^{3}H]rolipram were measured in each sample by Scatchard and linear regression analyses of specific binding. Results of saline- and imipramine-treated animals were compared in each region with a two-tailed Mann-Whitney test with exact inference. In addition, the effect of imipramine administration across the three brain regions was studied on (*R*)-[^{11}C]rolipram and [^{3}H]rolipram binding with multivariate analysis of variance. SPSS version 13.0 (SPSS, Chicago, IL) was used for the statistical analyses. Values of $P < 0.05$ were considered significant but were not corrected for multiple comparisons.

RESULTS

In vivo measurement of (*R*)-[^{11}C]rolipram binding with PET

V'_T of (*R*)-[^{11}C]rolipram was calculated by simultaneously measuring the radioactivity in the brain with a small animal PET scanner and serial (*R*)-[^{11}C]roli-

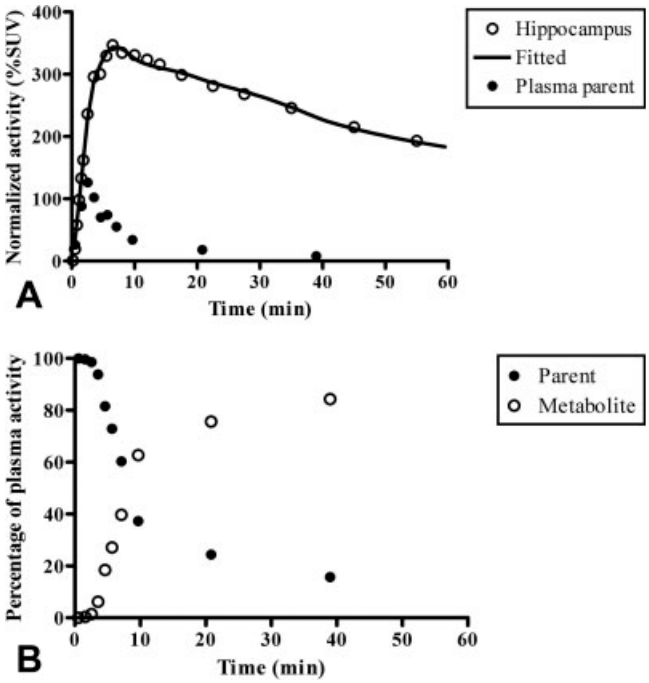

Fig. 2. Nonlinear least squares fitting using an unconstrained two-compartment model for hippocampus (**A**) and plasma metabolite data (**B**) from the imipramine-treated rat shown in Figure 1. From brain (open circles) and arterial plasma (closed circles) data, the two-compartment model provided good fitting (solid line) and gave results of $K_1 = 1.27$ ml/min/cm^3, $k_2 = 0.27$ /min, $k_3 = 0.087$ /min, $k_4 = 0.038$ /min, and $V'_T = 15.6$ ml/cm^3 (A). Plasma activity of the parent ((*R*)-[^{11}C]rolipram) and a hydrophilic metabolite was measured using radio-HPLC by obtaining 10 arterial blood samples in each experiment (B). Plasma parent levels shown in panel (A) was calculated as plasma activity (not shown) × percentage of parent (B).

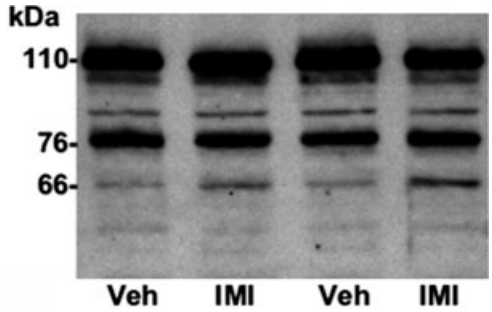

Fig. 3. Western blot analysis of PDE4A in the frontal cortex. Rats were administered vehicle (saline) or imipramine as described in the methods and levels of PDE4A were determined by Western blot analysis. Shown are representative lanes from vehicle and imipramine treated animals. The location of the PDE4A bands corresponding to PDE4A1 (66 kDa) and PDE4A5/A10 (110 kDa) are indicated and were determined to be PDE4A isoforms because of the size and because they were deleted in PDE4A null mice. The 76 kDa band was also deleted in PDE4A null mice, although it has not been previously described.

*TABLE I. PET measurement of (*R*)-[^{11}C]rolipram total distribution volume (V_T, ml/cm^3)*

	Group	
Region	Saline	Imipramine
Frontal cortex	67 ± 5	63 ± 5
Hippocampus	56 ± 6	51 ± 4
Diencephalon	48 ± 5	45 ± 4

V_T = binding potential + nondisplaceable activity; $n = 5$ in each group (Mean ± SEM).

TABLE II. In vitro B_{max} (fmol/mg protein) and K_d (nM) of [^{3}H]rolipram measured in the membrane fraction

	Group	
Region	Saline	Imipramine
B_{max}		
Frontal cortex	412 ± 33	378 ± 37
Hippocampus	397 ± 34	334 ± 31
Diencephalon	478 ± 45	381 ± 22
K_d		
Frontal cortex	10.2 ± 0.1	9.6 ± 0.6
Hippocampus	10.1 ± 0.7	9.9 ± 0.4
Diencephalon	12.3 ± 0.6	9.9 ± 0.3[a]

$n = 5$ in each group (Mean ± SEM).
[a] $P = 0.016$; $P = 0.032$ for a group difference in K_d across the three regions.

pram levels in arterial plasma (Fig. 2). Our previous study showed that ~86% of V'_T represents specific (i.e., displaceable) binding to PDE4 (Fujita et al., 2005). Similar to the previous study, V'_T was well identified using an unconstrained two compartment model showing %COV of 9.8 ± 1.5, 7.1 ± 1.0 and 6.6 ± 0.9 in frontal cortex, hippocampus, and diencephalon, respectively.

As described in the Materials and Methods section, f_1 was measured in separate sets of saline- and imipramine-treated animals to calculate V_T ($=V'_T/f_1 = BP$ + nondisplaceable activity), which is comparable to the in vitro measurement by homogenate binding assays. In addition to the purpose of obtaining a comparable outcome measure, we measured f_1 because concomitant medications such as imipramine can affect binding of (*R*)-[^{11}C]rolipram to plasma proteins. There was no significant difference in f_1 between the saline (30.5 ± 1.4%) and imipramine (30.7 ± 0.7%) groups. By using average f_1 values of each group, V_T was calculated (Table I). Saline- and imipramine-treated animals showed similar values in the three regions, with no significant differences either in each or across the three regions. Because ~86% (*R*)-[^{11}C]rolipram was specific binding and there was no difference in mass dose, these results indicate that there was no difference in BP of (*R*)-[^{11}C]rolipram measured in vivo.

In vitro measurement of [^{3}H]rolipram binding with homogenate binding assays

Brain samples were obtained from frontal cortex, hippocampus, and diencephalon at the end of each PET scan. Binding of [^{3}H]rolipram was measured in membrane (Table II) and cytosolic fractions (Table III). Among these multiple measurements in each region

TABLE III. In vitro B_{max} (fmol/mg protein) and K_d (nM) of [^{3}H]rolipram measured in the cytosolic fraction

Region	Group	
	Saline	Imipramine
B_{max}		
Frontal cortex	318 ± 65	370 ± 41
Hippocampus	173 ± 24	231 ± 26
Diencephalon	209 ± 26	249 ± 27
K_d		
Frontal cortex	13.2 ± 1.9	12.5 ± 0.6
Hippocampus	12.9 ± 1.3	13.6 ± 0.8
Diencephalon	12.2 ± 0.5	12.0 ± 0.7

$n = 5$ in each group (Mean ± SEM).

TABLE IV. Estimation for in vitro binding potential (ml/g tissue) of (R)-rolipram in the whole tissue

Region	Group	
	Saline	Imipramine
Frontal cortex	15.3 ± 0.7	15.4 ± 0.9
Hippocampus	12.3 ± 0.6	11.7 ± 0.4
Diencephalon	13.3 ± 0.9	13.3 ± 0.6

$n = 5$ in each group (Mean ± SEM).

(three regions × B_{max} and K_d values × membrane and cytosolic fractions × saline vs. imipramine), only one showed a moderate change: 19% decrease in K_d of the membrane fraction in diencephalon in imipramine compared to saline group ($P = 0.016$). This P-value would not survive correction for multiple comparisons. Multivariate analysis of variance showed a significant decrease in K_d of the membrane fraction across the three regions ($P = 0.032$ for the group effect) but did not detect a significant change in other parameters.

PET experiments measured rolipram binding in whole tissue and not in membrane or cytosolic. To compare comparable values measured in vivo and in vitro, the in vitro *BP* values for whole tissue were estimated first in the unit of ml/mg protein as the weighted average of the membrane and cytosolic fractions using the results shown in Tables II and III. The weighting was done based on total protein amount of the two tissue fractions in each region. Then *BP* of the whole tissue in the unit of ml/g tissue was calculated using the protein concentration. (*R*)-rolipram has 20 times greater affinity than the *S*-enantiomer (Schneider et al., 1986), and the *R*-enantiomer and racemic mixture were used in PET and homogenate binding assays, respectively. Therefore, in vitro *BP* values of the *R*-enantiomer were estimated by multiplying the *BP* values (ml/g tissue) of racemic mixture by a factor of 2. These estimated values are shown in Table IV. Whole tissue *BP* measured in vitro did not show a significant difference between the two groups either in each or across the three regions. Therefore, neither in vivo (PET, Table I) or in vitro (homogenate binding assays, Table IV) measurement showed a significant difference in *BP* of rolipram between the two groups.

TABLE V. Changes of PDE4 immunoreactivity after imipramine administration

Isozyme	Size[a] (isoform)	Changes (%)	
		Frontal cortex	Hippocampus
4A	66 (A1)	21.1 ± 6.9	−2.0 ± 4.5
	76[b]	0.7 ± 3.2	−0.7 ± 1.4
	110 (A5/A10)	−0.5 ± 2.7	−0.6 ± 1.8
4B	60 (B2)	8.1 ± 5.0	−1.4 ± 0.8
	110 (B3)	9.4 ± 8.1	−1.2 ± 1.5
	120 (B1)	2.4 ± 2.4	0.7 ± 2.0
4D	68 (D1/D2)	−9.3 ± 7.1	−5.0 ± 4.9
	93 (D3)	−9.5 ± 6.8	−5.0 ± 5.5
	105 (D5)	−5.6 ± 6.8	2.8 ± 4.5
	119 (D4)	14.1 ± 5.8	1.0 ± 4.9

$n = 6$ in each group (Mean ± SEM).
[a]Sizes given are in kilodaltons.
[b]This band has not been reported in previous studies but was identified as a PDE4A isoform that was deleted in PDE4A null mutant mice (Drs. D'Sa and Duman, unpublished data).

Immunoblot analysis of PDE4 isoforms

Literature on the selectivity of rolipram among PDE4 isoenzymes has been inconsistent, with reports showing smaller (Wang et al., 1997) and greater inhibition at PDE4D (Gale et al., 2003) compared to other isoforms, while a study showed similar inhibition at all four isoforms (Saldou et al., 1998). In addition, each PDE4 subfamily is composed of several isoforms, giving a total of at least 19 catalytically active isoforms (O'Donnell and Zhang, 2004). Therefore, rolipram binding may not show changes if some isozymes or splice variants increase and others decrease. To study possible changes in each of PDE4A, B and D isoforms, we performed Western blotting using isoform-selective antibodies. Three isoforms were measured for each of PDE4A and 4B, and four were measured for PDE4D (Fig. 3, Table V). Among these multiple measurements, no isoform showed a significant change. Only PDE4A1 (66 kDa) showed a trend of 21% increase in imipramine compared to saline groups, with $P = 0.065$.

DISCUSSION

The primary motivation of this study was to perform PET imaging using (*R*)-[^{11}C]rolipram in rats chronically treated with imipramine with the expectation of confirming most, but not all, prior postmortem in vitro studies demonstrating increased PDE4 levels. However, we found no significant change in brain uptake of this PDE4 PET radioligand with this antidepressant treatment. To address potential confounding technical problems, the study incorporated in vitro and careful in vivo analysis. For example, the study used a fully quantitative PET method with serial arterial blood samples that corrects for any changes in blood flow or metabolism of the tracer. We also confirmed the in vivo imaging studies with in vitro binding of membrane and cytosolic enzyme as well as immunoblot measures of the 10 isoforms.

Finally, the plasma free fraction of (*R*)-[^{11}C]rolipram was also measured to correct for any potential antidepressant-induced change in protein binding. With these internal controls and complementary assessments, we can fairly confidently say that chronic antidepressant administration via continuous minipump causes no significant increase of rolipram binding or PDE4 protein levels.

Although the homogenate binding assays detected a decrease in K_d of the membrane fraction in diencephalon of imipramine-treated animals and there was also a decrease in K_d of the membrane fraction across the three regions, there were no other changes in B_{max} or K_d in the homogenate binding. By using the same parameter of BP measured in the whole tissue, neither in vivo (PET, Table I) nor in vitro (homogenate binding assays, Table IV) measurement showed a significant effect of imipramine administration. The lack of changes in rolipram binding was supported by the results of Western blotting showing no significant change in any isoform. However, the trend of the increased PDE4A1 protein levels in frontal cortex detected in the current study are consistent to a certain degree with the results of a recent study on fluoxetine and ECS-induced changes in PDE4A mRNA splice variants (D'Sa et al., 2005). In that study, in frontal cortex, PDE4A1 increased but PDE4A5 and PDE4A10 did not. A larger sample size may be required to detect an increase in PDE4A protein levels in frontal cortex. The changes in hippocampus were not consistent between the two studies because the entire hippocampus was studied in the current study, whereas distinct subregions were studied in the previous study. Small–moderate changes in a single isoform would not be detectable with radiolabeled rolipram, either in vivo or in vitro, because of its no selectivity among isozymes and isoforms.

In general, the results of the current study are not consistent with most other reports on the effects of chronic antidepressant treatment on PDE4. Because various markers such as isoforms, splice variant mRNAs, or rolipram binding were measured using different antidepressant paradigms, it is difficult to discuss these studies together. However, other studies generally reported increases of 20% to >100% in these markers of PDE4 (Suda et al., 1998; Takahashi et al., 1999; Ye et al., 1997, 2000; Zhao et al., 2003a). There are a couple of methodological differences between those and the current study. The length of antidepressant treatment was up to 14 days in most other studies, whereas we treated for 20 or 27 days. A clear difference is the route of antidepressant administration. To avoid injection-induced stress and fluctuations of imipramine and the metabolite levels, we administered imipramine using osmotic minipump while all studies listed above administered antidepressants by repeated i.p. injection. In fact, the only prior study that used an osmotic minipump (Miro et al., 2002) had significantly different results from those of other studies (Suda et al., 1998; Takahashi et al., 1999; Ye et al., 1997, 2000; Zhao et al., 2003a). Miró et al. reported that chronic fluoxetine administration decreased rolipram binding in frontal and frontoparietal cortices and increased it in a few other discrete areas. The osmotic minipump produces relatively stable plasma levels, whereas repeated injections will have peak and trough plasma concentrations, which would be more extreme in animals with more rapid metabolism than in humans. Furthermore, repeated i.p. injections are clearly stressful to the animal, and antidepressant-induced upregulation of PDE4 may only occur in the presence of stress.

We measured rolipram binding both in vivo (PET) and in vitro (homogenate binding assays). These dual measurements were performed because the phosphorylated and activated form of PDE4 has greater sensitivity to rolipram inhibition (Hoffmann et al., 1998; Sette and Conti, 1996) and because PDE4's interactions with the in vivo milieu may affect the affinity of rolipram for PDE4 (Houslay and Adams, 2003). PET measurements should more accurately reflect the phosphorylation status and local environment of PDE4 than homogenate binding assays performed in an artificial medium. However, it should be noted that the current PET measurement did have a confounding factor of isoflurane anesthesia. The current study did not show a difference in BP between saline- and imipramine-administered rats with either PET or homogenate binding. However, the PET measurement of V_T (= binding potential plus nondisplaceable activity) (Table I) was about four times greater than the estimated in vitro BP of whole tissue (Table IV). Because our previous study has shown that ~86% of V_T is specific binding, that is, binding potential, the inclusion of nondisplaceable activity in the PET measurement can cause a ~14% difference but should not cause a 4-fold difference. Differences in PDE4's interactions with the in vivo milieu vs. an in vitro artificial medium may be a source of the discrepancies between the two measurements. In fact, even by comparing in vitro assays, K_d of whole tissue was different from an average of K_d obtained from the membrane and cytosolic fractions processed separately (Zhao et al., 2003b). Other possible sources of discrepancies are temperature (in vivo: 37°C; in vitro 30°C) and isoflurane anesthesia used in the PET experiments. Although to our knowledge, no study reported influence of isoflurane anesthesia on PDE4, one mouse study showed interactions between xylazine/ketamine anesthesia and PDE4 (Robichaud et al., 2002). Finally, PDE4 may be phosphorylated to a greater degree in vivo than in vitro and may have higher affinity to rolipram, which is equivalent to a decrease of K_d and an increase in *BP*.

Some studies using autoradiography detected discrete changes of PDE4 mRNA expression in small areas such as subregions of hippocampus and selected thalamic nuclei (D'Sa et al., 2005; Miro et al., 2002). The current study was limited by the spatial resolution of the PET device of ~1.7 mm full width half maximum (Johnson et al., 2002; Liow et al., 2003). Because one of the goals was to compare in vivo and in vitro measurements, comparably sized samples were used for PET, in vitro receptor binding, and Western blotting. To achieve this goal, changes of PDE4 in small discrete areas may have been overlooked in the current study.

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